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Expression of the short stature homeobox gene *Shox* is restricted by proximal and distal signals in chick limb buds and affects the length of skeletal elements

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Abstract

SHOX is a homeobox-containing gene, highly conserved among species as diverse as fish, chicken and humans. *SHOX* gene mutations have been shown to cause idiopathic short stature and skeletal malformations frequently observed in human patients with Turner, Leri–Weill and Langer syndromes. We cloned the chicken orthologue of *SHOX*, studied its expression pattern and compared this with expression of the highly related *Shox2*. *Shox* is expressed in central regions of early chick limb buds and proximal two thirds of later limbs, whereas *Shox2* is expressed more posteriorly in the proximal third of the limb bud. *Shox* expression is inhibited distally by signals from the apical ectodermal ridge, both Fgfs and Bmps, and proximally by retinoic acid signaling. We tested *Shox* functions by overexpression in embryos and micromass cultures. *Shox*-infected chick limbs had normal proximo-distal patterning but the length of skeletal elements was consistently increased. Primary chick limb bud cell cultures infected with *Shox* showed an initial increase in cartilage nodules but these did not enlarge. These results fit well with the proposed role of *Shox* in cartilage and bone differentiation and suggest chick embryos as a useful model to study further the role of *Shox* in limb development.

Keywords: Shox; Chick; Limb; Proximo-distal axis; Chondrogenesis; Micromass culture; Embryonic development

Introduction

Mutations within the human homeobox gene *SHOX* have been associated with short stature and the skeletal deformities found in Turner, Leri–Weill and Langer syndromes (reviewed in Blaschke and Rappold, 2000, 2006). In these syndromes, the long bones of the forearms and lower legs are disproportionately shortened and some patients exhibit Madelung deformity, which is characterized by a dorsal subluxation of the distal ulna (Leri and Weill, 1929). In human embryos, the *SHOX* gene is expressed, both in early limb development in the mid-region of the limb and later during skeletogenesis, in strikingly specific patterns (Clement-Jones et al., 2000). The SHOX protein is also present in both 12-week fetal and early childhood growth plate chondrocytes (Munns et al., 2004). These expression patterns suggests that *SHOX* plays several distinct roles in limb development including proximo-distal patterning and skeletal growth which together could account for the phenotype in human patients. Here we explore the roles of *Shox* in the developing limb bud of chick embryos.

Skeletal elements along the proximo-distal axis of the limb develop sequentially as the limb bud grows out. This outgrowth is maintained by the apical ectodermal ridge, a thickened epithelium at the distal tip of the limb bud. When the apical ridge is removed in chick embryos, outgrowth ceases resulting in a truncated skeleton (Saunders, 1948; Summerbell et al., 1973). Fibroblast growth factors (Fgfs; Niswander and Martin, 1993; Fallon et al., 1994) and bone morphogenetic proteins

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(Bmps; Francis et al., 1994) are expressed in the apical ridge and are involved in promoting distal outgrowth of the limb bud. Furthermore, there is evidence that Fgf signaling is antagonized by retinoic acid signaling from the proximal region of the limb (Mercader et al., 2000) and two antagonistic gradients of retinoic acid and Fgfs provide the information for proper patterning along the main body axis (Diez del Corral et al., 2003). As a consequence of these patterning signals, transcription factors are expressed in different domains along the proximo-distal axis of the limb bud with *Hoxa13* and *Hoxd13* being expressed distally and *Meis1* and *Meis2* proximally. Evidence from knockout mice suggests that *Hoxa13* and *Hoxd13* govern development of distal limb structures (Wellik and Capecchi, 2003) while work from chick embryos suggests that *Meis1* and *Meis2* govern development of proximal structures. Thus, when *Meis* genes are ectopically expressed using RCAS, the resulting limb skeleton is disrupted and smaller with the affected elements being in lower arm/leg and hand/foot plate whereas the upper arm/leg is normal (Mercader et al., 1999; Capdevila et al., 1999).

Proximo-distal patterning establishes the number and identity of skeletal elements in the different regions of the limb. The elements arise by mesenchymal cells forming condensations, which then differentiate into chondroblasts. The skeletal elements are first laid down in cartilage and high density primary cultures (micromasses) of limb bud mesenchyme cells are a good model to study these early steps of cartilage differentiation (Ahrens et al., 1977). The cartilage skeletal elements that will form the long bones then undergo rapid elongation initially through chondrocyte proliferation and matrix production; later, ossification takes place and further elongation is mediated by the activity of cartilaginous growth plates, which form at each end of the bones (reviewed by Kronenberg, 2003). Many of the same molecules are involved in early limb patterning and skeletogenesis. For example, Fgfs have a role in the formation of the condensations and Bmps are involved in regulating the size and growth of the condensations and the transit to differentiation (reviewed Hall and Miyake, 2000; Mariani and Martin, 2003). Ihh coordinates chondrocyte proliferation, chondrocyte differentiation and osteoblast differentiation. Sox9 is essential for converting cells of condensations into chondrocytes by stimulating the expression of cartilage matrix genes *Col2a1*, *Col11a2* and *Aggrecan* (reviewed by Kronenberg, 2003).

Here we examine the expression of *Shox* using chick embryos and compare it to the highly homologous gene *Shox2*. Both are present in human and chicken but no *SHOX* orthologue has been found in rodents (Blaschke et al., 1998; Clement-Jones et al., 2000). Recently, the *Shox*-related *Shox2* gene has been genetically removed from the developing limb buds of mice (Cobb et al., 2006), which do not have the *Shox* gene. These mice have severely short limbs due to virtual loss of the element in the upper arm and leg. This is unlike human patients that have mutations in the *SHOX* gene, where the middle part of the limbs is affected. In human patients, short stature has never so far been associated with mutations for *SHOX2*. We then investigated the regulation of *Shox* expression in chick limbs and its function by overexpressing the gene in early limb buds. Finally, we tested the effects of overexpressing *Shox* on cartilage formation and differentiation in micromass cultures.

Materials and methods

Cloning of the chicken Shox cDNA

The full-length coding sequence of the chicken *Shox* cDNA was assembled from two overlapping PCR fragments generated with the primers CE2 for 5′-CCG TGT GGA TAG CAG CGC G-3′/CE3rev 5′-CTG CAC CCT AGC TTC AGA CAG TC-3′ and CE3 for 5′-GAT CTA CGA GTG CAA GGA GAA GC-3′/CE6rev 5′-GCG TGT CAG AGC CCC AGG GCC-3′, respectively. These cDNA fragments were joined via a unique EclHKI site in a pBLUESCRIPT vector (Stratagene) and the open reading frame was verified by sequence analysis of both strands.

Embryos

Fertilized White Leghorn chicken eggs were obtained from H. Stewart (Lincolnshire) for *in situ* hybridization, cultures and manipulations. Standard pathogen-free eggs (spf) from Lohmann Tierzucht (Germany) were used for virus infections. Eggs were incubated at 37°C and staged according to Hamburger and Hamilton (1951).

In situ hybridization

Whole mount *in situ* hybridization on chick embryos was performed as previously described (Wilkinson and Nieto, 1993). Probes were synthesized using a standard protocol for *Shox*, *Shox2* (EST 603126448F1 from ARK-Genomics; Boardman et al., 2002), *Meis1* (Mercader et al., 1999), *Hoxd11*, *Hoxa13* (Nelson et al., 1996), *Gag* (Hughes et al., 1997), *Ihh* (Vortkamp et al., 1996), *MyoD* (Rudnicki et al., 1993), *Sox9*, *Type II Collagen* and *Aggrecan* (a kind gift from Prof Hurle). *In situ* hybridization on micromass cultures was performed as described (Chimal-Monroy et al., 2003).

Micromass culture

Micromass cultures were prepared from chick limb buds (stages 20–21) embryos (see Vogel and Tickle, 1993). A 10-μl drop containing 2×10^5 cells was placed into the center of each well of a 4 or 24 well dish (Nunc). After 1 h, 500 μl of culture medium was added (Dulbecco's Modified Eagle Medium (DMEM): F12 formulation, 50/50 (Gibco BRL) with 10% fetal calf serum (FCS), 1% glutamine and 1% antibiotic/antimycotic). Cultures were maintained in a 37°C incubator gassed with 5% CO_2, fed every 24 h and cultured for three to seven days.

Tissue and bead grafts

All manipulations were performed using stages 19–21 chick embryos. The apical ridge was either ablated using sharp needles or an additional ridge was grafted to the dorsal surface of a host limb bud and pinned with wire staples. Staples were made from nickel-chromium wire, ϕ 25 μm (Goodfellow Metals Ltd, England). Dorsal ectoderm was removed from wing buds using 1:2000 Nile blue to stain the ectoderm; the desired region was then peeled off. For bead implantation, a small slit was made in the dorsal side of limb bud, or just under the apical ridge at the desired position and a bead was implanted; alternatively, the bead was held in place on the surface of the limb bud with a staple (as above).

The following beads, proteins and chemicals were used: (1) Heparin beads (Sigma) were soaked in 1 mg/ml Fgf4 or Fgf8 (R&D systems) in PBS with 0.1% BSA. (2) Heparin beads were soaked in 0.01 mg/ml Bmp solutions (Genetics Institute) diluted down from stocks with PBS/0.1% BSA. (3) Heparin beads were soaked in 1 mg/ml Noggin protein (R&D systems). (4) Affigel blue beads (Biorad) were soaked in 8–10 mg/ml Sonic Hedgehog (R&D systems). (5) Formate derivatized AG1-X2 beads (Biorad) were soaked in 0.1, 0.5 or 1 mg/ml retinoic acid (Sigma) diluted in DMSO from a stock concentration of 10 mg/ml.

Retrovirus construction and infection

Full-length chicken *Shox* was cloned into the Slax13 shuttle vector using *Nco*I and *Bam*HI. This plasmid was digested with *Cla*I and cloned into the replication competent avian specific retroviral construct (A coat protein subtype; RCAS(A)). This retrovirus was termed RCAS-*Shox*. DF1 cells were transfected with DNA for RCAS-*Shox* or an RCAS(A) construct expressing enhanced green fluorescent protein (RCAS-Egfp). Medium was harvested and virus concentrated to give a titer of 1×10^8 virus particles/ml. Chick embryos were infected by injecting concentrated virus into the right coelom at stages 13–14. Micromass cultures were infected by adding 1 μl concentrated virus (or 1 μl culture medium for control cultures) to the culture at the time of plating.

Western Blot

Control DF1 cells or DF1 cells transfected with RCAS-*Shox* were isolated 48 h after transfection in RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% Sodium deoxycholate, 0.1% SDS and freshly added inhibitors, 10 μg/ml pepstatin A, leupeptin and chymostatin (Peptide Inc) and 100 g/ml phenyl methyl sulfonyl fluoride (Sigma). Protein concentration was assessed using Coomassie protein reagent (Pierce) and 50 μg protein was loaded per well; membranes were blotted with anti-Shox antibody (1:2000; Rao et al., 2001) and anti-rabbit HRP-labeled secondary antibody (1:5000; Sigma) or with anti-actin antibody (1:1000; Sigma) and anti-mouse HRP-labeled secondary antibody (1:5000; Sigma).

Immunohistochemistry

Micromass cultures were fixed 15 min in 2% paraformaldehyde, washed three times 5 min in phosphate-buffered saline (PBS), blocked 45 min in DMEM, with 10% FCS, 0.2% Triton X-100 (termed MST) at room temperature, incubated 1 h in MF20 primary antibody (Developmental Studies Hybridoma Bank, Iowa) diluted 1:5 in MST, washed two times quickly and two times 5 min in PBS. They were then incubated 30 min with secondary anti-mouse antibody (Sigma), washed as above, incubated 30 min in Vectastain ABC reagent, washed as described above, incubated with DAB substrate kit for peroxidase (Vector Laboratories, SK-4100) and when desired level of staining was reached, reaction was stopped by washing with water. Cultures were stored in PBS.

Tunnel staining was performed on micromass cultures following the protocol of the In Situ Cell death detection kit, Fluorescein (Roche nr 1684795) following the protocol for adherent cells. For BrdU staining, cultures were incubated for 1 h in a final concentration of 1 μM BrdU (Roche). Staining was performed using undiluted G3G4 antibody (Developmental Studies Hybridoma Bank, Iowa) following standard techniques (Gunhaga et al., 2000).

Alcian green staining

Embryos were fixed in 5% trichloroacetic acid (TCA) at day 9 of incubation and stained for cartilage using 0.1% Alcian Green in 70% acetic acid, dehydrated in ethanol and cleared in methyl salicylate.

Alcian blue–alizarin red staining

Embryos were fixed in ethanol for 1 week, post-fixed in acetone for a week, stained in 0.015% Alcian blue, 0.005% alizarin red in 70% ethanol for a week, cleared in 20% glycerol, 0.1% KOH for three days and then put through successive washes of glycerol up to 100% glycerol. Skeletal elements were measured using Openlab 4.0.1 and *t* test was performed using SygmaStat 3.1.

Results

Shox expression in chick embryos

The chicken *Shox* gene (GenBank accession DQ675517) is located on chromosome 1 and encodes a protein of 243 amino acids that exhibits 94% homology to the human SHOX protein (85% at the nucleotide level; Rao et al., 1997). All known functional domains including the homeodomain, SH3 binding domain and the OAR domain are identical between human and chicken suggesting functional conservation (Fig. 1).

We analyzed *Shox* expression in chick embryos at different stages (18–36) of development. The first sign of *Shox* expression in limb-forming regions was observed at stages 18–19. At this stage *Shox* is weakly expressed both in wing and leg buds with stronger expression at the posterior side of leg buds. *Shox* also appears to be expressed in visceral arches and neural tube (Fig. 2A). At stages 20–22, *Shox* is strongly expressed in the central mesoderm of the limb bud, surrounded by a rim of non-expressing cells distally and at anterior and posterior margins and there is a relatively sharp boundary proximally (Figs. 2B and C). Sections of these whole mounts show that *Shox* is expressed uniformly throughout the central mesoderm but not in the ectoderm (data not shown). The pattern of expression in the visceral arches at these stages is similar to

Protein Alignment of Human and Chicken SHOX

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SHOX      1 MEELTAFVSKSFDQKSKDGNGGGGGGGGKKDSITYREVLESGLARSRELGTSDSSLQDIT
Shox      1 MEELTAFVSKSFDQKSKE-SGGGGGGGNKKETITYREVLESGLARSRELGNSDSALPDMT
            ****************   ******* **  ****************** *** * * *

SHOX     61 EGGGHCPVHLFKDHVDNDKEKLKEFGTARVAEGIYECKEKREDVKSEDEDGQTKLKQRRS
Shox     60 EGSNHCPVHLFKDHVESDKDKLKEFAAGRTAEGIYECKEKREDVKSEDEDGQTKLKQRRS
            *** ***********  ** *****   * ******************************

SHOX    121 RTNFTLEQLNELERLFDETHYPDAFMREELSQRLGLSEARVQVWFQNRRAKCRKQENQMH
Shox    120 RTNFTLEQLNELERLFDETHYPDAFMREELSQRLGLSEARVQVWFQNRRAKCRKQENQMH
            ************************************************************

SHOX    181 KGVILGTANHLDACRVAPYVNMGALRMPFQQVQAQLQLEGVAHAHPHLHPHLAAHAPYLM
Shox    180 KGVILGTASHLDACRVAPYVNMGALRMPFQQVQAQLQLEGVAHAHPHLHPHLAAHAPYLM
            ******** ***************************************************

SHOX    241 FPPPPFGLPIASLAESASAAAVVAAAAKSNSKNSSIADLRLKARKHAEALGL
Shox    240 FPPPPFGLPIASLAESASAAAVVAAAAKSNSKNSSIADLRLKARKHAEALGL
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Fig. 1. Comparison of human SHOX and chick Shox protein sequence. *Indicates amino acid homology, the homeobox is shown in red, a putative SH3 binding domain in green and the OAR domain in blue.

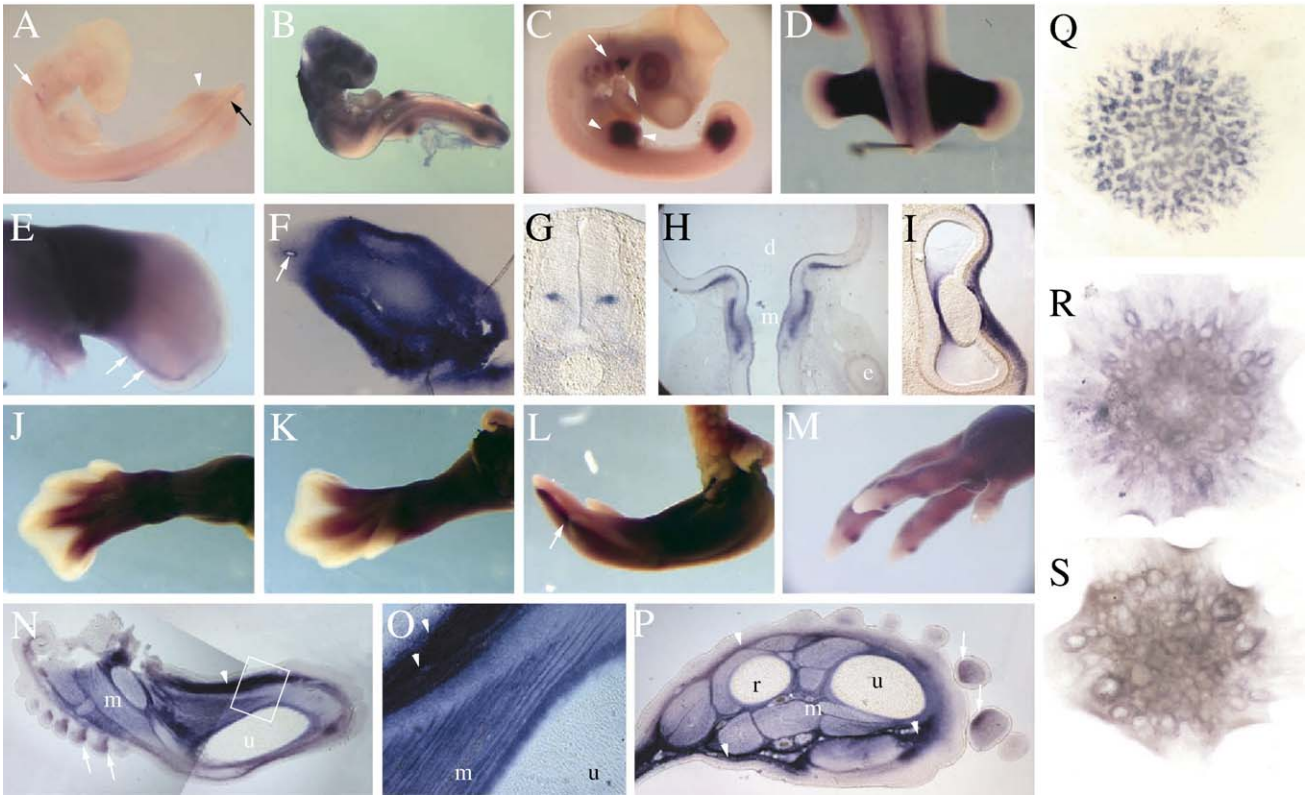

Fig. 2. Expression of *Shox* in chick embryos (A) Stage 19; low level *Shox* expression in several regions including branchial arches (white arrow), hindlimb buds (white arrowhead), neural tube (black arrow). (B) Stage 20; strong expression in head and limb buds. (C) Stage 22; *Shox* expression in central region of limb bud surrounded by rim of non-expressing cells (arrowheads) and in central region of branchial arches (arrow). (D) Stage 25; *Shox* expression restricted to proximal two thirds of buds, expression absent at tips. (E) Stage 25 leg; higher power; *Shox* expression in vasculature (arrows), at posterior of bud. (F) Section of stage 25 leg; expression in mesenchyme of proximal two thirds of limb bud, with lower level expression in areas where cartilage and muscles will form. *Shox* also expressed in marginal vein (arrow). (G) Stage 25; section through neural tube; strong expression at boundary between ventricular zone and zone of presumptive motor neurons. (H) Stage 25; sagittal section of head; *Shox* expression in brain at posterior margin of the diencephalon (d) and metacoele (m); (e) eye. (I) Stage 25; section of the eye, expression is seen in the mesenchyme overlying the eye, staining behind the lens (l) is trapping (J, K) Stage 29 leg; strong *Shox* expression begins to appear along sides of digital rays towards tip on dorsal (J) but not ventral (K) side. (L) Stage 34 wing; strong expression on dorsal side of hand plate (note marked boundary; arrow). (M) Stage 36 leg; *Shox* expression on both dorsal and ventral sides of digits and in scales. (N) Stage 36 upper wing; low magnification of section; *Shox* expression outlines muscles (m), ulna (u), in featherbuds (arrows) and in layer under the dermis (arrowhead). (O) High power of area outlined in N showing stripes of expression in muscles, strong expression under dermis (arrowhead) and ulna. Note absence of expression in cartilage. (P) Transverse section of stage 36 wing; *Shox* expressed around radius (r) and ulna, muscles, under dermis (arrowhead) and in featherbuds (arrows). Note expression beneath dermis is stronger ventrally. (Q) *Shox* expression in micromasses cultured for 3 days associated with condensing cartilage nodules. (R, S) At 4 and 5 days of culture, expression more clearly restricted to the periphery of nodules and absent from center.

that of the limb buds, in that *Shox* transcripts are only found in the central regions of the facial processes and not at the margins (Fig. 2C).

At stages 24–25, *Shox* is expressed throughout the proximal region of the limb bud but not distally (Figs. 2D and E). Sections of whole mounts show that *Shox* expression is reduced in the region where cartilage is condensing (Fig. 2F). Expression can also be detected in the vasculature of both wing and leg buds, particularly clearly in the posterior of the limb buds (Fig. 2E, arrows). In whole mount sections, *Shox* can be seen to be expressed in the marginal vein (Fig. 2F; arrow). At these stages, *Shox* is also expressed on the inside of the mandibular primordia (data not shown), in bands along the neural tube, particularly strongly at the boundary of ventricular and presumptive motor neuron zones in the area that contains p2 ventral neural progenitors (Fig. 2G). Expression is also seen in the brain around the outer surface of the posterior diencephalon and metacoele (Fig. 2H) and around the eye (Fig. 2I). At stages 26–29, *Shox* expression in both wings and legs is similar to that seen at stage 24/25.

At stage 29, *Shox* expression begins to appear more distally, along the digital rays, on the dorsal side of both wings and legs (compare Figs. 2J and K; dorsal and ventral respectively). At stage 34, *Shox* becomes more widely expressed dorsally in the wing and there is a clear boundary at the dorso-ventral margin (Fig. 2L; arrow). In the leg at stages 35–36, strong *Shox* expression can be seen in the scales ventrally on the foot and around the digits (Fig. 2M). In sections, expression can be seen in featherbuds (Figs. 2N and P; arrows), in the layer of mesenchyme just below the dermis (Figs. 2N–P; arrowheads), and outlining both the muscles and cartilage elements in upper and lower arm (Figs. 2N–P). It should be noted that the expression in the dermis is stronger ventrally than dorsally (Fig. 2P).

To study further the association with cartilage formation, *Shox* expression was examined in micromass cultures of mesenchyme cells from stage 20 to 22 limb buds. At three days of culture (Fig. 2Q), scattered patches of *Shox* transcripts were found in the central region of the micromass where cartilage nodules form. As the nodules developed, it became clear that *Shox* expression was in rings, this suggests that *Shox* is restricted to the periphery of the nodules and absent from the center (day 4 and 5 of culture; Figs. 2R and S).

Shox2 expression in chick embryos

Comparable with the situation in humans, *SHOX* has a close homologue, *SHOX2,* in chicken, which is located on chromosome 9. To investigate possible overlaps in the expression of *Shox* and *Shox2* that may complicate the interpretation of the effects of manipulating *Shox* expression in chick embryos, we compared *Shox* and *Shox2* expression during chick limb development.

At stage 19, no *Shox2* expression can be seen in the limb buds but staining is detected in the sinus venosus (Figs. 3A and A′; arrow). By stage 21, the expression in the sinus venosus has become stronger and additional staining can be seen in the limb buds, where staining is excluded from a rim around the edge of the limb bud, which is wider anteriorly and distally (Fig. 3B). By stage 23, expression in the limb buds is even more restricted to the posterior region. The expression domain in the leg buds is smaller than in the wing buds and staining is also observed in somites, atria and sinus venosus (Figs. 3C and C′). As development progresses, the *Shox2* expression domain becomes more confined to the proximal posterior part of the limb bud (Figs. 3D and E). By stage 30, expression is still seen in the heart and this postero-proximal domain in the limbs but expression then reappears in the medial region of leg and wing, and in the hand and footplate (Fig. 3F); this appears to be in the tissue surrounding the muscles and in the tendons in the handplate. Sections of stage 23 embryos show *Shox2* expression throughout the mesenchyme of the proximal part of the limb bud (Fig. 3G), in a punctate pattern in the neural tube with also more widespread low level expression throughout the tube, in the dorsal root ganglia (Fig. 3H) and in the heart (Fig. 3I). At stage 27 *Shox* expression is around the dorsal-proximal muscles of the limb (Fig. 3J) and apparently not around the cartilage rudiments.

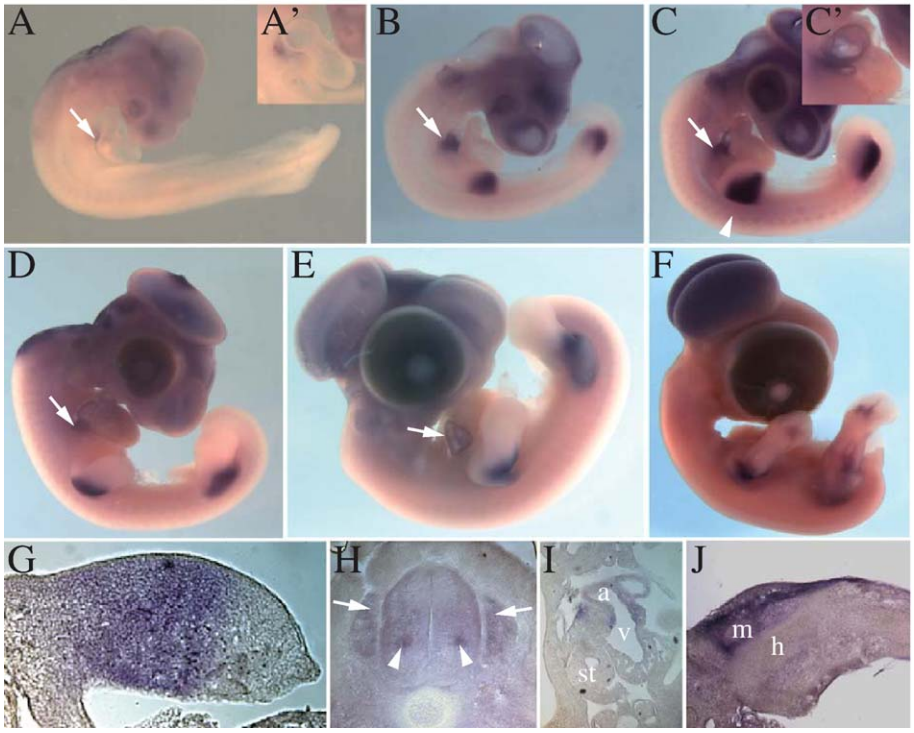

Fig. 3. Expression of *Shox2* in chick embryos. (A) Stage 19; *Shox2* expressed in sinus venosus (arrow). (A′) Larger magnification of the heart (B) Stage 21; *Shox2* expression in sinus venosus (arrow) and limb bud where *Shox2* is present throughout the limb bud surrounded by a rim of non-expressing cells that is wider at the anterior and distal margin. (C) Stage 23; expression in the limb bud in a central region with non-expressing cells distal and anterior, in the dorsal root ganglia (arrowhead), sinus venosus (arrow) and faint staining can also be seen in atria (C′). (D) Stage 25; expression in the sinus venosus (arrow), atrium and the proximal third of the limb bud in the posterior half. (E) Stage 27; *Shox2* expression is in the atria of the heart (arrow) and in the postero-proximal region of the limb bud. (F) Stage 30; *Shox2* expression is in the postero-proximal region but also in the lower leg and hand- and footplate. (G) Section of stage 23 limb bud showing expression throughout the proximal mesenchyme. (H) Section of stage 23 neural tube showing two spots of expression (arrowhead) and throughout dorsal root ganglia (arrow). (I) Section of stage 23 heart showing expression of *Shox2* in the tissue connecting atrium and the ventricle (st) stomach, (v) ventricle, (a) atrium. (J) Section of stage 27 limb showing *Shox2* expression around muscle masses dorsal to humerus (h), (m) muscle.

Regulation of Shox expression in early chick limb bud stages

To explore the regulation of *Shox* expression in early chick limb buds, the effects of a number of signaling molecules known to regulate expression of other genes involved in developing limb buds were studied.

We investigated the possibility that signals from the apical ectodermal ridge might be involved in inhibiting *Shox* expression in distal mesenchyme. When the apical ridge was removed from chick wing buds, *Shox* expression was found to extend to the tip 24 h later ($n=5$; Fig. 4A). To verify that this extension is not simply due to death of the non-expressing cells at the limb bud tip, we tested the effects of grafting an additional ridge onto the dorsal surface of chick wing buds over the region where *Shox* is normally expressed ($n=11$; Figs. 4B and C; see arrows). Twenty-four hours after grafting, *Shox* expression had decreased in the mesenchyme (Fig. 4B; arrow) and at 36 h, when the extra ridge had induced a new "mini" limb, *Shox* expression could be seen proximally in the outgrowth although the tip still lacked *Shox* expression (Fig. 4C; arrow). Taken together, these results demonstrate that *Shox* expression is negatively regulated by signaling from the apical ridge.

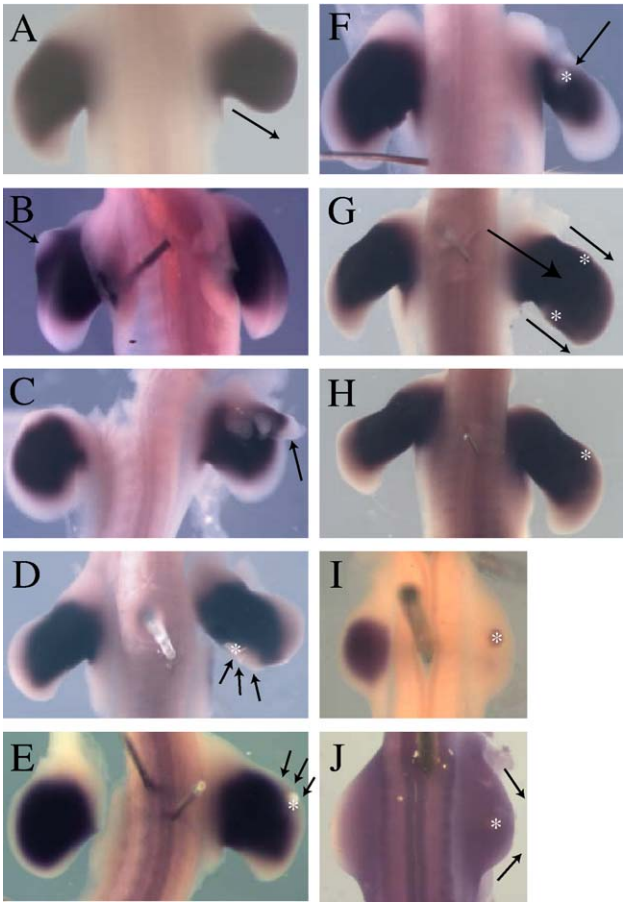

Fig. 4. Regulation of *Shox* expression in chick wing buds. Dorsal views in all panels except B, which is ventral view. Right limb bud manipulated; left limb bud serves as control. (A) 24 h after ridge removal *Shox* expression extends to wing bud tip (arrow). (B, C) Grafting an additional ridge to the dorsal side of the wing inhibits *Shox* (arrow). *Shox* expression also inhibited by beads soaked in Fgf4 (D), Fgf8 (E) and Bmp4 (F) whereas beads (*) soaked in Noggin (G) extended *Shox* expression to tip (arrows). Implanting a bead (*) soaked in Shh (H) had no effect on *Shox* expression even though the limb bud was wider. Beads (*) soaked in retinoic acid reduce *Shox* expression (I) but extend *Meis1* expression (J; arrows) compare with left control limb with distal rim not expressing *Meis1*.

We then defined which ridge signals inhibit *Shox* expression by grafting beads soaked in Fgfs and Bmps. Fgfs are expressed in the apical ridge and beads soaked in either Fgf4 ($n=10$; Fig. 4D; see arrows) or Fgf8 ($n=8$; Fig. 4E; arrowed) stapled onto the dorsal side of the wing bud led to local reduction of *Shox* expression at 24 h. Bmps are expressed both in the AER and underlying distal mesenchyme. Beads soaked in Bmp2 ($n=7$), Bmp4 ($n=6$; Fig. 4F) or Bmp7 ($n=7$) implanted to the anterior margin of the limb led to extensive cell death but also to local reduction of *Shox* expression in some cases (see arrows; Fig. 4F). To test further whether Bmp signaling is involved in regulating *Shox*, beads soaked in the Bmp inhibitor, Noggin, were grafted to the anterior and posterior margin of wing buds ($n=5$; Fig. 4G; arrows). Twenty-four hours after implanting Noggin beads, *Shox* expression clearly extends right to the tip of the limb. These results show that the non-expressing rim of *Shox* expression at the distal tip of the limb buds is maintained by both Fgfs and Bmps.

Another important signal in proximo-distal patterning is retinoic acid. Therefore, we tested whether *Shox* expression is controlled by retinoic acid signaling by grafting beads soaked in different concentrations of retinoic acid (0.1, 05 and 1 μg/μl) into early (Stage 19) wing buds. Six hours following implantation of retinoic acid beads, *Shox* expression was dramatically reduced especially at the higher concentrations (0.1 μg/μl RA $n=2/4$; 0.5 μg/μl RA $n=2/4$; 1 μg/μl RA $n=7/7$; Fig. 4I). As previous work has shown that retinoic acid extends the expression of Meis1 to the distal tip, we compared the effect of retinoic acid on *Meis1* expression with that found on *Shox*. Indeed, after grafts of the same retinoic acid beads, the expression of *Meis1* extends right to the tip of the limb (0.1 μg/μl RA $n=2/2$; 0.5 μg/μl RA $n=3/3$; 1 μg/μl RA $n=7/7$; Fig. 4K; see arrows). We also tested the effect of retinoic acid on the *Shox* expression domain at longer time points (24 h) but the results were hard to interpret due to the limb truncations induced by retinoic acid beads placed apically. Therefore, to assess the effect of retinoic acid at later stages of development, we grafted beads to stage 26 limb buds at different positions along the proximal distal axis and studied *Shox* expression after 6 h. Retinoic acid beads implanted distally or medially in the limb did not alter *Shox* expression ($n=3$ and $n=2$, respectively) but when grafted proximally, *Shox* expression was reduced ($n=2$; data not shown). These results suggest that, unlike *Meis1* expression, *Shox* expression is inhibited by retinoic acid signaling in the most proximal part of the limb bud.

Finally, other crucial signaling molecules involved in limb development were also investigated. Both implanting beads soaked in Sonic hedgehog (Shh) ($n=6$; Fig. 2H) at the anterior margin of the limb or removing dorsal ectoderm ($n=8$), which is known to express *Wnt7a*, did not change expression of *Shox* (data not shown). This suggests that *Shox* expression does not depend on Shh or Wnt7a signaling.

Overexpression of Shox in chick limb buds using a replication competent avian specific retrovirus

To study the function of *Shox* in limb development, the chicken *Shox* sequence was cloned into a replication competent avian specific retroviral (RCAS) vector and viral particles were produced in DF1 cells and then concentrated to a titer of 1×10^8 viruses/ml. To verify that the virus produced the Shox protein, total protein from DF1 cells transfected with RCAS-*Shox* was extracted and a Western Blot was performed using an anti-Shox polyclonal antibody. In extracts of cells transfected with RCAS-*Shox*, a 32-kDa band was detected whereas no band was seen in extracts from uninfected DF1 cells (Fig. 5A).

Concentrated *Shox* virus was injected into the coelom of stages 13–14 spf chicken embryos, in the region which will give rise to the right wing; left wings were uninfected and served as controls. RCAS-*Egfp* was injected into the coelom of stages 13–14 spf chicken embryos to act as a control for virus infection. Both 3 days after infection and at 10 days of total development, all embryos injected with RCAS-*Egfp* had infection throughout the right wing ($n=5/5$ and $n=8/8$, respectively) and partial infection in the leg too ($n=3/5$ and $n=4/8$, respectively) In addition, whole mount *in situ* hybridization for *Shox*, performed 48 h after infection, showed that *Shox* was expressed throughout the right wing and leg (and in the heart; $n=3$; Fig. 5B′) whereas left wing and leg showed a normal pattern of *Shox* expression (Fig. 5B).

At days 9–10 (Figs. 5C and C′; $n=30$) and 13 (Figs. 5D and D′; $n=24$) of development, *Shox*-infected embryos did not show gross morphological changes in skeletal pattern of either wings (Figs. 5C and D) or legs (Figs. 5C and D′). However, in some day 13 embryos ($n=3/24$), a delay in ossification of the coracoid in the shoulder girdle (Fig. 5D; arrow) was observed. In addition, one of these embryos also exhibited a delay in ossification of the ischium of the right pelvic girdle (Fig. 4D′; arrow). We then studied the skeletal elements in more detail and measured the total length of skeletal elements of the wing (humerus, radius, ulna and metacarpal of digit three) and leg (femur, tibia and fibula) of 5 embryos infected with RCAS-*Egfp* and 10 embryos with RCAS-*Shox*. The ratio between right and left leg/wing was then calculated to take into account any differences in stages of development. In the control RCAS-*Egfp*-infected embryos, the mean ratio for all measurements was about 1 (Fig. 5E shows values for wing skeletal elements). In contrast, the mean ratio for the length of *Shox*-infected right wing elements versus the length of control left wing elements was consistently greater then 1 for all elements measured. Comparison between ratios for *Shox*-infected wings and *Egfp*-infected wings using *t* tests showed that these increases in the ratios were significant for humerus ($P<0.044$), radius ($P<0.047$) and metacarpal of digit three ($P<0.002$). The increase for the ulna ($P<0.323$) was not significant. For the length of the leg elements; although the ratios were greater than one, in no case was this increase significant. We then measured the length of the ossified region of the wing and leg elements and compared these. A significant increase was noticed in the ratio of the length of the ossified portion of the humerus in *Shox*-infected versus *Egfp*-infected embryos ($P<0.044$) but no significant difference in extent of ossification was found for any other ossified region examined.

To look for possible patterning defects, we studied expression of genes involved in proximo-distal patterning, cartilage and muscle differentiation. *In situ* hybridization for *Hoxa13*, *Hoxd11*, *Meis1*, *Sox9* and *MyoD* were carried out 3 days after injection. *Hoxa13* was expressed in the same pattern at the tip of both right and left wing buds ($n=3$; Fig. 5F) and *Meis1* was expressed proximally to the same extent in both infected and control wings ($n=6$; Fig. 5G). No changes in expression of either *Hoxd11* ($n=4$; data not shown), *Sox9* in cartilage condensations ($n=3$; Fig. 5H) or *MyoD* ($n=3$; Fig. 5I) in the presumptive muscle cells could be detected. We also examined the effect of overexpression of *Shox* on *Shox2* expression but could not see a noticeable change in the *Shox2* expression pattern ($n=4$; Fig. 5J), which was still restricted to the proximal third of the limb bud in a posterior domain.

Overexpression of Shox in primary chick limb bud cell cultures using RCAS

To study directly the effect of *Shox* on cartilage differentiation, we infected micromass cultures of mesenchyme cells from chick limb buds (stages 20–22) with RCAS-*Shox* or with RCAS-*Egfp* by adding virus at the time the cells were plated and then monitoring effects on chondrogenesis. At 3 days, cartilage nodules had formed in virus-infected cultures and control cultures. The overall nodular pattern was similar in control uninfected cultures, cultures infected with RCAS-*Egfp* and cultures infected with RCAS-*Shox* (RCAS-*Egfp* not shown; Figs. 6A and D). To assess the extent of chondrogenesis in the cultures, the number of nodules was counted in cultures made on two separate occasions (total number of cultures counted for control $n=4$; RCAS-*Egfp* $n=5$; RCAS-*Shox* $n=6$; Experiment 1: control uninfected 55/55/54 nodules; RCAS-*Egfp* 48/54/49 nodules; RCAS-*Shox* 64/72/67 nodules; Experiment 2: control 101 nodules; RCAS-*Egfp* 92/80 nodules RCAS-*Shox* 137/112/121 nodules). These data show that there was an increase in nodule number in cultures infected with RCAS-*Shox* (23% more nodules compared to uninfected control cultures). By 4 days, the nodules in control or RCAS-*Egfp*-infected cultures had enlarged significantly and stained more strongly than nodules in RCAS-*Shox*-infected cultures in which there still appeared to be more nodules (Figs. 6B and E). Five days after RCAS-*Shox* infection, cartilage was present widely throughout the whole culture but the nodules had not enlarged. Furthermore, Alcian blue staining was less intense in the nodules compared to control cultures in which the nodules had enlarged and now stained more intensely with Alcian blue (Figs. 6C and F). This difference was still detectable at six and seven days of culturing cells infected with RCAS-*Shox* (data not shown).

We also examined expression of *Sox9*, *Ihh, Col2* and *Aggrecan*, as the products of these genes are involved in the formation of cartilage condensations and later involved in the

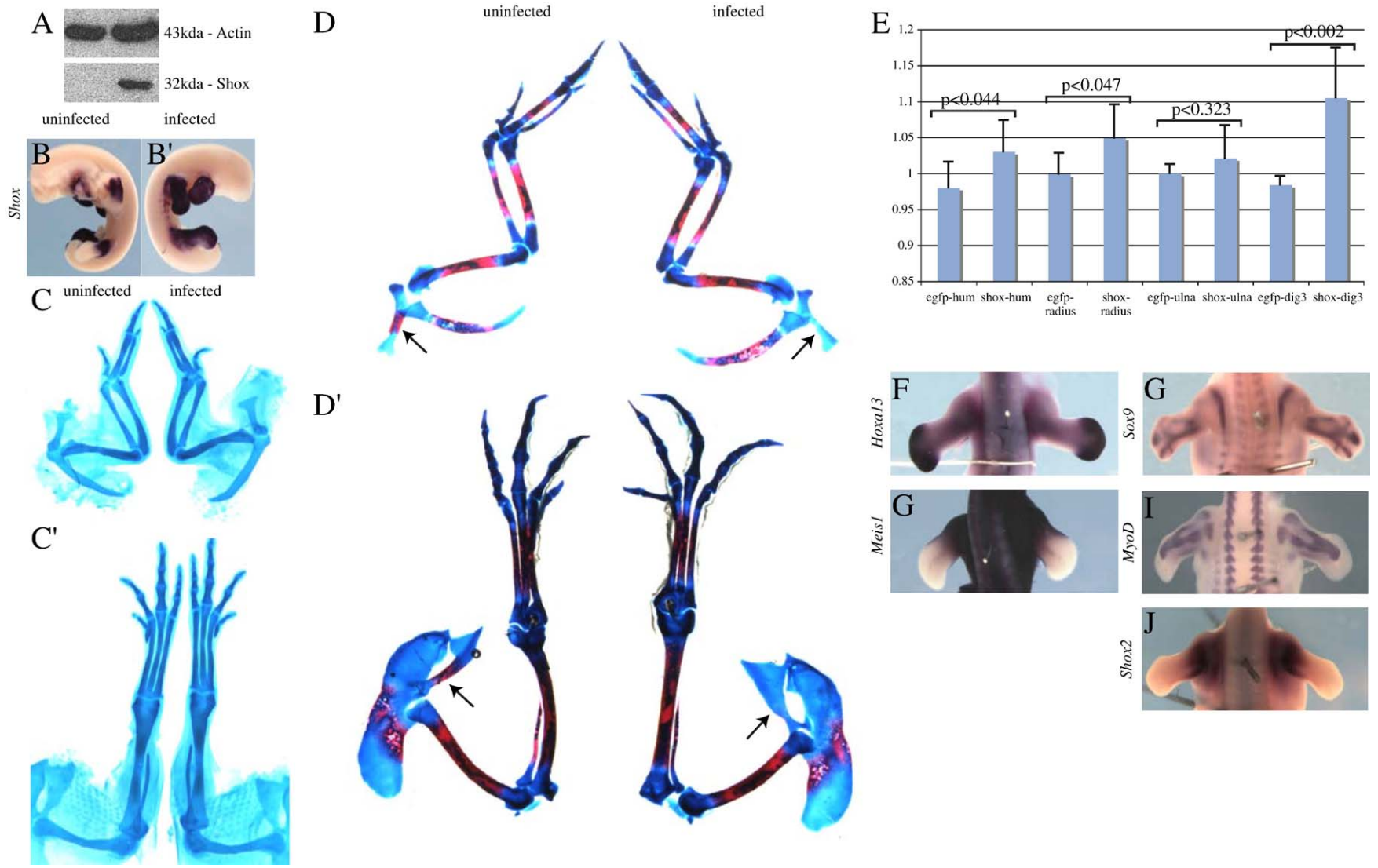

Fig. 5. Effect of overexpressing RCAS-*Shox* in chick wings. (a) Western blot showing a 43-kDa band for actin in extracts from both uninfected and infected DF1 cells and a 32-kDa Shox band in extracts from infected but not in uninfected DF1 cells. (B–D, G–I) Uninjected control wings or legs shown on left; RCAS-*Shox*-infected wings or legs on right. (B and B′) *Shox* expression shows efficient infection. Left side of embryo (B) showing normal *Shox* expression, whereas right side (B′) shows overexpression throughout wing, heart, most of the leg and some of the flank. At 10 days of incubation, no change in skeletal pattern was observed in either wings (C) or legs (C′). Skeletal elements stained with Alcian green. At 13 days of incubation, no change in skeletal pattern could be observed in either wings (D) or legs (D′). Skeletal elements stained with Alcian blue and alizarin red. Note delay in ossification of the coracoid and the ischium of infected right limbs (arrows in D and D′) compared with control left limbs. (E) Ratios of the length of right versus left humerus, radius, ulna or metacarpal of digit three in 13-day-old embryos infected with RCAS-*Egfp* or RCAS-*Shox*. *P* value obtained after performing *t* test to compare ratios from *Shox* and *Egfp*-injected embryos is shown above brackets. Forty-eight hours after infection with RCAS-*Shox*, no change in expression of *Hoxa13* (F) and *Meis1* (G), *Sox9* (H), *MyoD* (I) and *Shox2* (J) could be detected.

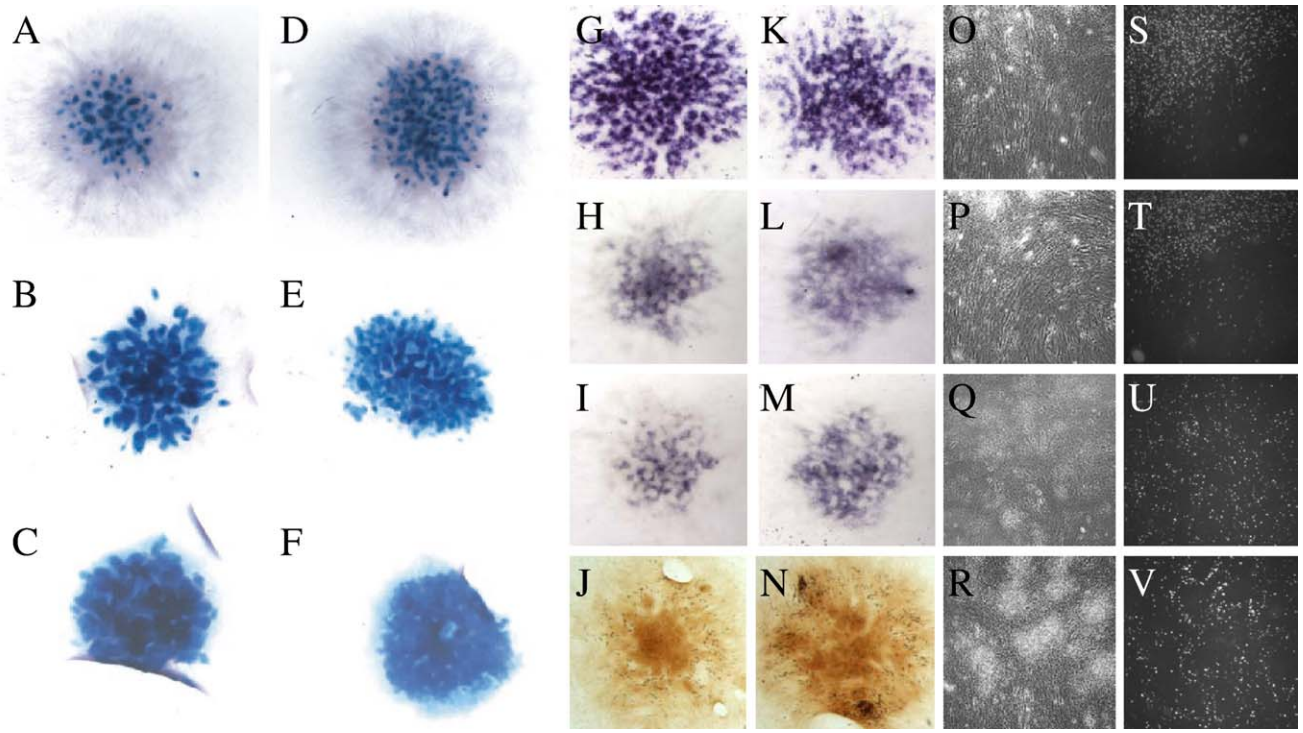

Fig. 6. Effects of overexpressing *Shox* in micromass culture. (A–F) Alcian blue staining of micromasses, control uninfected cultures (A–C) and RCAS-*Shox*-infected cultures (D–F). At 3 days of culture both uninfected and infected cultures look similar, although note increase in number of nodules in RCAS-*Shox*-infected cultures (compare A and D). 4 day (B, E) and 5 day (C, F) cultures. Clear enlargement of nodules with intensified Alcian blue staining in control cultures. In RCAS-*Shox*-infected cultures (compare E and F with B and C, respectively), cartilage staining more widespread but less intense, nodules not enlarged. (G and K) *Sox9* expression at 3 days of culture. Decrease in *Sox9* expression in cultures infected with RCAS-*Shox* (compare K with G). (H and L) *Collagen2* expression at 3 days of culture. *Collagen2* expression appears more widespread but less punctate in cultures infected with RCAS-*Shox* (compare L with H). (I and M) *Aggrecan* expression at 3 days of culture. *Aggrecan* expression is more widespread in cultures infected with RCAS-*Shox* (compare M with I). (J) Control Culture; (N) culture infected with RCAS-*Shox*; MF20 antibody to show muscle cells. More myogenic cells are present in *Shox*-infected cultures at 3 days. (O, P bright-field and S, T fluorescence) BrdU incorporation after 1 h at 3 days of culture, no difference detected between *Shox*-infected (T) and control (S) cultures. (Q, R bright-field and U, V fluorescence) TUNEL staining at 3 days of culture, no difference detected between *Shox*-infected (V) and control (U) cultures.

correct execution and maintenance of cartilage differentiation program (reviewed by Kronenberg, 2003). Three days after infection with RCAS-*Shox*, expression of *Sox9* (Figs. 6G and K) and *Ihh* (data not shown) appeared reduced in comparison to expression in control cultures. The region of the culture in which *Col2* is expressed is large in infected cultures compared to control cultures but the clear punctate staining in control cultures is less pronounced in cultures infected with RCAS-*Shox* (Figs. 5H and L). A similar effect was seen on the expression levels of *Aggrecan* as this is present in a larger part of the culture after infection with RCAS-*Shox* (Figs. 6I and M). We monitored the level of BrdU incorporation to find out whether the decrease in level of expression was due to cell cycle arrest in cells infected with *Shox*. We detected no difference in overall labeling between control and RCAS-Shox-infected cultures when measuring overall level of fluorescence in random images taken of stained control uninfected and *Shox*-infected cultures. For BrdU, we compared 24 images from 6 uninfected control cultures with 28 images from 7 *Shox*-infected cultures, these cultures were generated on three separate occasions (Figs. 6O and S compared to Figs. 6P and T). We also performed TUNEL staining to examine the level of apoptosis and noticed no change in overall fluorescence in RCAS-*Shox*-infected cultures compared to control cultures. We compared 19 images from 6 uninfected control cultures with 17 images from 5 *Shox* infected, these cultures were generated on two separate occasions. (Figs. 6Q and U compared to Figs. 6R and V).

Finally, we examined differentiation of muscle cells in the primary chick limb bud cell cultures by staining with MF20 antibody, which detects myosin heavy chain. At 3 days of culture, an increase in the initial number of differentiating muscle cells was observed after infection with RCAS-*Shox* (Figs. 6J and N).

Discussion

We have cloned the chicken *Shox* gene and shown that it is widely expressed in the limb bud but then becomes restricted to the proximal two-thirds of the limb. This pattern of expression is consistent with the fact that these regions of the lower arms are the areas affected in human patients. At later stages, *Shox* is also expressed in connective tissue around cartilage elements and muscles and in a layer of cells beneath the dermis. *Shox* is also expressed in other regions of the chick embryo including branchial arches, nervous system and vasculature. In contrast, *Shox2* is expressed, posteriorly in the proximal third of the limb bud, the sinus venosus and transiently in the somites. This

expression pattern nicely corresponds with the observed expression in human embryos (Clement-Jones et al., 2000). Thus, *Shox* expression overlaps with *Shox2* only in the most proximal part of the limb bud suggesting that chicken lend themselves well as a model to dissect Shox and Shox2 functions. In this study, we have concentrated on the analysis of *Shox*. We show that, in the early limb buds, *Shox* expression is negatively regulated by both Bmps and Fgfs distally and by retinoic acid proximally. *Shox* overexpression has no detectable effect on proximo-distal pattern of skeletal elements but increases the length of skeletal elements and occasionally the timing of ossification is altered. In micromass cultures, overexpression of *Shox* affects formation and growth of cartilage nodules.

There are strong similarities between the expression of *Shox* in chick and human embryos but our analyses also reveals important novel aspects. In human embryos, *SHOX* expression starts at Carnegie stage (CS) 13 (28 days), and in chicken embryos at an equivalent stage (Hamburger 18–19). However, in the chick limb bud, *Shox* is expressed throughout the proximal two-thirds, whereas *SHOX* in human embryos appears to be more restricted to the middle part of the limb bud. In chick limbs, *Shox* expression is associated with developing muscle and cartilage elements whereas in human limbs, although *SHOX* expression has been observed in association with skeletal elements, no association with the developing muscle has been observed so far (Clement-Jones et al., 2000). The analysis of *Shox* expression in the early chicken embryo also revealed some other new sites of expression in the vasculature and nervous system that had not been described in human embryos (Clement-Jones et al., 2000). In particular, the fact that *SHOX* is expressed in the vasculature could be relevant to the lymphedema seen in some of the *SHOX* deficient patients.

If *Shox* is involved in specification of the 'middle' segment of the limb, we would expect its expression to be regulated similarly to other genes implicated in proximo-distal patterning, such as *Meis*, *Hoxa* and *Hoxd* genes. The *Shox* expression domain overlaps with the expression domain of *Meis1* and *Meis2* but extends more distally; whereas *Meis* expression extends more proximally. *Hoxa13* and *Hoxd13* expression initially overlap with *Shox* expression in the early limb bud but later become complementary as *Hoxa13* and *Hoxd13* are expressed in the tip, which does not express *Shox*. Expression of *Hoxa13* and *Hoxd13* at the limb bud tip depends on Fgf signaling (Vargesson et al., 2001) and at stage 25/26 is inhibited by retinoic acid (Hayamizu and Bryant, 1994) whereas expression of *Meis1* and *Meis2* in the proximal limb bud depends on retinoic acid signaling and is inhibited by Fgfs and Bmps (Capdevila et al., 1999; Mercader et al., 2000). Interestingly, we observed that *Shox* expression is similarly inhibited by Fgfs and Bmps. Thus, the same signals prevent expression of both *Meis* genes and *Shox* at the tip of the limb. Bmps are expressed not only in the apical ridge but also by distal limb mesenchyme and it is not clear whether it is ridge-derived or mesenchyme-derived Bmp signals or both that contribute to the regulation of *Shox* expression. We also found that retinoic acid inhibited *Shox* expression, suggesting that it is important to restrict Shox expression from the most proximal part of the limb bud. From these experiments, we thus conclude that *Shox* is carefully restricted to the proximal-medial region by signals operating both distally and proximally.

When *Meis1* or *Meis2* is overexpressed in chick wings, proximal structures develop normally but distal structures are affected and proximalized (Mercader et al., 1999; Capdevila et al., 1999). From the medially restricted *Shox* expression pattern, we expected that overexpressing this gene throughout the limb, including the handplate where *Shox* is not normally expressed, might also lead to alterations in proximo-distal skeletal patterning. However, we detected no gross changes in proximo-distal pattern following overexpression of *Shox*. Rao et al. (2001) showed in a study using cell lines that *SHOX* function depends on cell type. They found that SHOX can translocate to the nucleus in all cell types studied, but only observed activation of transcription in an osteogenic cell line. Thus, co-factors may be required for *SHOX* to function in limb patterning.

In human patients, the growth of the long bones is most affected in the forearms and lower legs. In our experiments, in which we overexpress *Shox*, we found significant increases in the lengths of skeletal elements. The humerus, radius and metacarpal of digit three were longer after infection with RCAS-Shox. In addition, the length of the ossified part of the humerus was also increased due to ectopic Shox expression indicating that Shox controls the length of bones, independent of their position. Human patients with multiple copies of the *SHOX* gene exhibit tall stature and our results may resemble this phenotype (Kanaka-Gantenbein et al., 2004). It is interesting to note that the increase in length of skeletal elements in limbs where *Shox* is overexpressed is most significant ($P>0.002$) for the metacarpal of digit three. We also analyzed the effects of *Shox* overexpression in micromass cultures, which are model systems for the initial steps of chondrogenesis (Ahrens et al., 1977). At 3 days of incubation, overexpression of *Shox* promoted chondrogenesis as evident from an increased expression of genes encoding cartilage matrix components such as *Col2* and *Aggrecan* and enhanced Alcian blue staining. At the same time *Sox9* and *Ihh* expression appeared to be decreased. This decrease in expression of genes that regulate chondrogenesis may presage the fact that in later *Shox*-infected micromasses, cartilage differentiation does not mature in the same way as in control micromasses. These results therefore clearly demonstrate a function of *Shox* during early steps of chondrogenesis.

Marchini et al. (2004) showed that overexpression of *SHOX* in primary chondrocytes and osteogenic stable cell lines induces cell cycle arrest and apoptosis in culture. In contrast, overexpression of *Shox* in chick limb bud micromass cultures did not appear to produce the same effect. Both BrdU incorporation and TUNEL staining was unaltered after *Shox* overexpression. These results once more emphasize the difference of *Shox* functions during early chondrogenesis and bone growth and might explain why some of the effects

of a SHOX deficiency in humans do not manifest itself until after birth (Ross et al., 2001).

Taken together, we have shown that *Shox* and *Shox2* are expressed in a similar fashion in chicken to that described in human embryos and demonstrated that *Shox* expression in the chick limb bud is regulated by both proximal and distal signals, leaving a medial expression domain that correlates well with the phenotype observed in human patients. In addition, the increase in length of skeletal elements obtained in chick limbs after overexpression might resemble the increased height of a patient carrying multiple copies of the *SHOX* gene. In summary, these results strongly suggest the chick embryo as a suitable model to further study the events in *SHOX*-related human disease.

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References

Ahrens, P.B., Solursh, M., Reiter, R.S., 1977. Stage-related capacity for limb chondrogenesis in cell culture. Dev. Biol. 60, 69–82.

Blaschke, R.J., Rappold, G.A., 2000. SHOX: growth, Leri–Weill and Turner syndromes. Trends Endocrinol. Metab. 11, 227–230.

Blaschke, R.J., Rappold, G., 2006. The pseudoautosomal regions, SHOX and disease. Curr. Opin. Genet. Dev. 16, 233–239.

Blaschke, R.J., Monaghan, A.P., Schiller, S., Schechinger, B., Rao, E., Padilla-Nash, H., Ried, T., Rappold, G.A., 1998. SHOT, a SHOX-related homeobox gene, is implicated in craniofacial, brain, heart, and limb development. Proc. Natl. Acad. Sci. U. S. A. 95, 2406–2411.

Boardman, P.E., Sanz-Ezquerro, J., Overton, I.M., Burt, D.W., Bosch, E., Fong, W.T., Tickle, C., Brown, W.R., Wilson, S.A., Hubbard, S.J., 2002. A comprehensive collection of chicken cDNAs. Curr. Biol. 12, 1965–1969.

Capdevila, J., Tsukui, T., Rodriquez Esteban, C., Zappavigna, V., Izpisua Belmonte, J.C., 1999. Control of vertebrate limb outgrowth by the proximal factor Meis2 and distal antagonism of BMPs by Gremlin. Mol. Cell 4, 839–849.

Chimal-Monroy, J., Rodriguez-Leon, J., Montero, J.A., Ganan, Y., Macias, D., Merino, R., Hurle, J.M., 2003. Analysis of the molecular cascade responsible for mesodermal limb chondrogenesis: Sox genes and BMP signaling. Dev. Biol. 257, 292–301.

Clement-Jones, M., Schiller, S., Rao, E., Blaschke, R.J., Zuniga, A., Zeller, R., Robson, S.C., Binder, G., Glass, I., Strachan, T., Lindsay, S., Rappold, G.A., 2000. The short stature homeobox gene SHOX is involved in skeletal abnormalities in Turner syndrome. Hum. Mol. Genet. 9, 695–702.

Cobb, J., Dierich, A., Huss-Garcia, Y., Duboule, D., 2006. A mouse model for human short-stature syndromes identifies Shox2 as an upstream regulator of Runx2 during long-bone development. Proc. Natl. Acad. Sci. U. S. A. 103, 4511–4515.

Diez del Corral, R., Olivera-Martinez, I., Goriely, A., Gale, E., Maden, M., Storey, K., 2003. Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron 40, 65–79.

Fallon, J.F., Lopez, A., Ros, M.A., Savage, M.P., Olwin, B.B., Simandl, B.K., 1994. FGF-2: apical ectodermal ridge growth signal for chick limb development. Science 264, 104–107.

Francis, P.H., Richardson, M.K., Brickell, P.M., Tickle, C., 1994. Bone morphogenetic proteins and a signalling pathway that controls patterning in the developing chick limb. Development 120, 209–218.

Gunhaga, L., Jessell, T.M., Edlund, T., 2000. Sonic hedgehog signaling at gastrula stages specifies ventral telencephalic cells in the chick embryo. Development 127, 3283–3293.

Hall, B.K., Miyake, T., 2000. All for one and one for all: condensations and the initiation of skeletal development. BioEssays 22, 138–147.

Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49–92.

Hayamizu, T.F., Bryant, S.V., 1994. Reciprocal changes in Hox D13 and RAR-beta 2 expression in response to retinoic acid in chick limb buds. Dev. Biol. 166, 123–132.

Hughes, E.S., Bell, J.E., Simmonds, P., 1997. Investigation of the dynamics of the spread of human immunodeficiency virus to brain and other tissues by evolutionary analysis of sequences from the p17gag and env genes. J. Virol. 71, 1272–1280.

Kanaka-Gantenbein, C., Kitsiou, S., Mavrou, A., Stamoyannou, L., Kolialexi, A., Kekou, K., Liakopoulou, M., Chrousos, G., 2004. Tall stature, insulin resistance, and disturbed behavior in a girl with the triple x syndrome harboring three SHOX genes: offspring of a father with mosaic Klinefelter syndrome but with two maternal x chromosomes. Horm. Res. 61, 205–210.

Kronenberg, H.M., 2003. Developmental regulation of the growth plate. Nature 423, 332–336.

Leri, A., Weill, J., 1929. Une affection congenetal et symetrique du developpement osseux: la dyschondrosteose. Bull. Mem. Soc. Med. Hop. Paris 35, 1491–1494.

Marchini, A., Marttila, T., Winter, A., Caldeira, S., Malanchi, I., Blaschke, R.J., Hacker, B., Rao, E., Karperien, M., Wit, J.M., Richter, W., Tommasino, M., Rappold, G.A., 2004. The short stature homeodomain protein SHOX induces cellular growth arrest and apoptosis and is expressed in human growth plate chondrocytes. J. Biol. Chem. 279, 37103–37114.

Mariani, F.V., Martin, G.R., 2003. Deciphering skeletal patterning: clues from the limb. Nature 423, 319–325.

Mercader, N., Leonardo, E., Azpiazu, N., Serrano, A., Morata, G., Martinez, C., Torres, M., 1999. Conserved regulation of proximodistal limb axis development by Meis1/Hth. Nature 402, 425–429.

Mercader, N., Leonardo, E., Piedra, M.E., Martinez, A.C., Ros, M.A., Torres, M., 2000. Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. Development 127, 3961–3970.

Munns, C.J., Haase, H.R., Crowther, L.M., Hayes, M.T., Blaschke, R., Rappold, G., Glass, I.A., Batch, J.A., 2004. Expression of SHOX in human fetal and childhood growth plate. J. Clin. Endocrinol. Metab. 89, 4130–4135.

Nelson, C.E., Morgan, B.A., Burke, A.C., Laufer, E., DiMambro, E., Murtaugh, L.C., Gonzales, E., Tessarollo, L., Parada, L.F., Tabin, C., 1996. Analysis of Hox gene expression in the chick limb bud. Development 122, 1449–1466.

Niswander, L., Martin, G.R., 1993. FGF-4 regulates expression of Evx-1 in the developing mouse limb. Development 119, 287–294.

Rao, E., Weiss, B., Fukami, M., Rump, A., Niesler, B., Mertz, A., Muroya, K., Binder, G., Kirsch, S., Winkelmann, M., Nordsiek, G., Heinrich, U., Breuning, M.H., Ranke, M.B., Rosenthal, A., Ogata, T., Rappold, G.A., 1997. Pseudoautosomal deletions encompassing a novel homeobox gene cause growth failure in idiopathic short stature and Turner syndrome. Nat. Genet. 16, 54–63.

Rao, E., Blaschke, R.J., Marchini, A., Niesler, B., Burnett, M., Rappold, G.A., 2001. The Leri–Weill and Turner syndrome homeobox gene SHOX encodes a cell-type specific transcriptional activator. Hum. Mol. Genet. 10, 3083–3091.

Ross, J.L., Scott Jr., C., Marttila, P., Kowal, K., Nass, A., Papenhausen, P., Abboudi, J., Osterman, L., Kushner, H., Carter, P., Ezaki, M., Elder, F., Wei, F., Chen, H., Zinn, A.R., 2001. Phenotypes associated with SHOX deficiency. J. Clin. Endocrinol. Metab. 86, 5674–5680.

Rudnicki, M.A., Schnegelsberg, P.N., Stead, R.H., Braun, T., Arnold, H.H., Jaenisch, R., 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75, 1351–1359.

Saunders Jr., J.W., 1948. The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. J. Exp. Zool. 108, 363–404.

Summerbell, D., Lewis, J.H., Wolpert, L., 1973. Positional information in chick limb morphogenesis. Nature 244, 492–496.

Vargesson, N., Kostakopoulou, K., Drossopoulou, G., Papageorgiou, S., Tickle, C., 2001. Characterisation of hoxa gene expression in the chick limb bud in response to FGF. Dev. Dyn. 220, 87–90.

Vogel, A., Tickle, C., 1993. FGF-4 maintains polarizing activity of posterior limb bud cells in vivo and in vitro. Development 119, 199–206.

Vortkamp, A., Lee, K., Lanske, B., Segre, G.V., Kronenberg, H.M., Tabin, C.J., 1996. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 273, 613–622.

Wellik, D.M., Capecchi, M.R., 2003. Hox10 and Hox11 genes are required to globally pattern the mammalian skeleton. Science 301, 363–367.

Wilkinson, D.G., Nieto, M.A., 1993. Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. Methods Enzymol. 225, 361–373.